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Supplementation with *N*-Acetyl Cysteine Affects Motor and Cognitive Function in Young but Not Old Mice

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ABSTRACT

Background: *N*-acetyl cysteine (NAC) is a thiolic antioxidant that is thought to increase cellular glutathione (GSH) by augmenting the concentration of available cysteine, an essential precursor to GSH production. Manipulating redox status can affect brain function, and NAC intake has been associated with improving brain function in models of neurodegenerative diseases.

Objectives: The objective of the study was to determine if short-term dietary supplementation with NAC could ameliorate functional impairment associated with aging.

Methods: C57BL/6J male mice aged 6, 12, or 24 mo were fed a control diet or the control diet supplemented with 0.3% NAC for a total of 12 wk. After 4 wk of dietary supplementation, mice began a series of behavioral tests to measure spontaneous activity (locomotor activity test), psychomotor performance (bridge-walking and coordinated running), and cognitive capacity (Morris water maze and discriminated active avoidance). The performance of the mice on these tests was analyzed through the use of analyses of variance with Age and Diet as factors.

Results: Supplementation of NAC improved peak motor performance in a coordinated running task by 14% ($P < 0.05$), and increased the time spent around the platform by 24% in a Morris water maze at age 6 mo. However, the supplementation had no to minimal effect on the motor and cognitive functions of 12- and 24-mo-old mice.

Conclusions: The findings of this preclinical study support the claim that NAC has nootropic properties in 6-mo-old mice, but suggest that it may not be useful for improving motor and cognitive impairments in older mice. *J Nutr* 2019;149:463–470.

Keywords: NAC, *N*-acetyl cysteine, aging, motor, cognitive

Introduction

N-acetyl cysteine (NAC) is an acetyl derivative of the amino acid L-cysteine and a precursor of glutathione (GSH). Although NAC is historically well known for its use in the treatment of paracetamol overdose (1, 2), it was first prescribed as a mucolytic agent (3). It is described by the WHO as an effective and safe medicine (4), and is inexpensive. More recently, due to this new role as an antioxidant, NAC has become a popular amino acid nutritional supplement that has been promoted for healthy aging, and is widely available in stores and online. Although it is a weak free radical scavenger, NAC's major antioxidative role stems from serving as a precursor to GSH synthesis by providing cysteine, a process that is the rate-limiting step of GSH biosynthesis (5). Furthermore, as a proton donor, NAC provides substrates for the activity of glutathione peroxidase (6). Based on its antioxidant capacity, other therapeutic uses of NAC have been considered.

The chemical properties of NAC suggest a therapeutic potential which has been explored by several studies of its supplementation alone or in combination with other compounds (5, 7). NAC administration has been studied in a variety of conditions such as nephropathy, HIV/AIDS, and heavy-metal toxicity (8, 9). Specifically, NAC intake has been studied for its effect on redox stress. In diabetic rat models, NAC administration reduced lipid peroxidation markers in liver and kidneys (10), enhanced glutathione peroxidase activity and normalized lipid hydroperoxides (11), and prevented high sucrose–induced protein oxidative damage (12). Pesticide toxicity studies have identified a protective role of NAC by reducing kidney and liver toxicity, and immunotoxicity (7).

Oxidative stress has been implicated as playing a role in age-related brain dysfunction (13, 14). Specifically, protein oxidation levels in specific brain regions have been associated with the severity of cognitive and psychomotor impairments

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(15). More recently, shifts in the redox state of glutathione have been used as a more sensitive indicator of oxidative stress (16). Pro-oxidizing shifts have been shown in brain regions (17), and have the ability to impair redox-dependent cellular processes and contribute to the accumulation of oxidative damage (18). Therefore, it seems that interventions manipulating the concentrations of glutathione could lead to improvements in motor and cognitive function. Supplementation with GSH or L-cysteine does not lead to any substantial increase in glutathione concentrations and is therefore not a fruitful avenue to use as an intervention to alleviate the effect of aging or neurologic deficits. Contrary to GSH or L-cysteine supplementation, NAC intake leads to increased plasma and brain GSH (19, 20), which makes NAC a more suitable supplement to alleviate the effects of aging. NAC's antioxidative properties, along with its interaction with inflammatory cytokines and neurotransmitters, have led to it increasingly being used to treat psychiatric disorders and drug addiction (9, 21–23). Studies have determined that NAC supplementation alone or in combination with other compounds (e.g., α-lipoic acid, α-tocopherol, thioproline) reduced the effect of age on ATPase activity and peroxidation in rat brain synaptosomes (24), protein damage in mouse synaptic mitochondria (25), and mitochondrial gene expression (26). A study in a model of premature aging, SAMP8 mice, reported that subcutaneous injections with 100 mg NAC/kg body weight for 4 wk led to improved cognition (20). Another study in a model of premature aging in males and females found a beneficial effect of NAC [combined with thioproline (another antioxidant and precursor of GSH)] on age-related impairments (27). Furthermore, NAC supplementation has also been associated with improvements in selective brain domains in an aging model (28). NAC supplementation improved spatial learning and memory in a mouse model of Alzheimer disease that used streptozotocin, (29), and improved depressive and motor deficits in a mouse model of Huntington disease (30, 31).

Results from these previous studies suggested that the supplementation of NAC may attenuate age-related functional declines; however, in many of these studies NAC was combined with other compounds or tested in a disease state with high amounts of redox stress. The main goal of this study was to address whether supplementation of NAC alone can reverse age-related impairments in motor and cognitive function in mice. Furthermore, the study was designed to determine whether the effect of NAC were age dependent by studying young and middle-age mice.

Methods

Mice and diet

All the procedures pertaining to animal handling and maintenance adhered to the NIH guidelines and were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee. Male C57BL/6J mice (n = 192) aged 6, 12, or 24 mo were obtained from the National Institute on Aging and subsequently maintained in the vivarium of the University of North Texas Health Science Center. Mice were housed in groups of 3–4 in clear polycarbonate cages at 23°C ± 1°C under a 12-h light/dark cycle starting at 0700, and had ad libitum access to food and water. After 1 wk of acclimation, the mice from each age group were randomly assigned to receive either a control diet (LabDiet, 5LG6, cat. no. 1813505 from TestDiet) or the control diet supplemented with 0.3% NAC (cat. no. 1804051 from TestDiet); both diets contained 21% protein; 13% fat; and 65% carbohydrates. This dose was chosen based on previous studies that used this dose and reported significant declines in protein oxidation in synaptic mitochondria, and because the supplementation partially restored age-related memory impairments (25, 28). Body weights were measured weekly and food intake was measured daily the week prior to behavioral testing. A total of 7 mice died during the study (one 12-mo-old control, three 24-mo-old control, and three 24-mo-old NAC). The mice were on their respective diets for a total of 10 wk, with behavioral testing starting after 4 wk of treatment.

Behavioral test battery

The behavioral test battery has been used previously to detect age-related declines in neurocognitive, psychomotor, and sensory functions. It was expected that mice of advanced age would exhibit impaired function relative to young mice, and that treatment with NAC could improve function over the course of treatment (32–35).

Motor function.

Spontaneous forward locomotion and rearing (standing) movements of the mice were monitored with the use of a Digiscan apparatus (Omnitech Instruments) during a 16-min session. Reflexive musculoskeletal responses of the mice were measured, including the ability to initiate walking, turn in a dead-end alley, exhibit negative geotaxis, grip a horizontal wire, and walk across narrow bridges of increasing difficulty. The mice were administered each of these tests during 4 consecutive daily sessions. The average distance travelled, rearing activity, and latency to walk, turn, tread, and fall were recorded. Motor learning and maximum performance on a coordinated running task were measured with the use of an accelerating rotorod.

Spatial learning and memory.

Competence of the mice to locate a hidden platform was measured in a series of 9 training sessions (1 session/d composed of 5 trials with an intertrial interval of 90 s). The learning index was calculated by averaging the path length to the platform from sessions 2–4. A probe trial (30 s) was conducted on the fifth trial of session 9, and a delayed probe trial was conducted 1 wk after the probe trial on session 9. The probe index was calculated by averaging the percent time spent in the target quadrant, the areas 20 and 40 cm around the target, and the target site during the 30-s probe trials for sessions 9 and 10.

Discriminated avoidance.

A T-maze rested on a grid floor wired to deliver a 0.69-mA scrambled shock to the feet. The discriminated avoidance test consisted of 3 sessions separated by 1 h. In each of the 3 sessions the mice were tested until they reached criterion of learning to make correct avoidances or completed a maximum of 25 trials.

Statistical analysis

The data were subjected to 2-way ANOVAs, with Age and Diet as between-group factors. Post-hoc comparisons were made through the use of single degree-of-freedom F tests in which the denominator was the error for the overall analysis. Water maze data were subjected to 3-way ANOVA, with Age and Diet as between-group factors, and session as within-group factor. An ANCOVA with body weights as the covariate was done for the coordinated running analysis. The α level was set at 0.05 for all analyses.

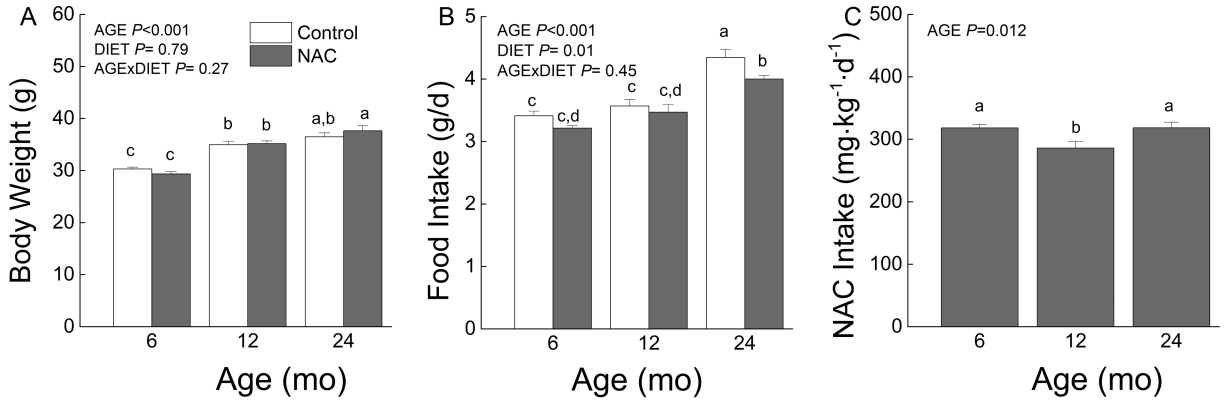

FIGURE 1 Effect of age and NAC supplementation on body weight (A), daily food intake (B), and daily NAC intake (C) in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM ($n = 29$–32). Labeled means without a common letter differ, $P < 0.05$. NAC, *N*-acetyl cysteine.

An ANCOVA with body weights at the time of coordinated running testing did not change the outcome of the ANOVA with a main effect of Age ($P < 0.05$), a main effect of Body Weight ($P < 0.05$), and the individual comparisons at 6 mo between control and NAC-supplemented mice remaining significant. The only change was that the difference between the 6- and 24-mo-old control mice was not significant ($P = 0.332$).

Results

Body weight, food intake, and NAC intake

Body weights (averaged across the study of the surviving mice only), food intake, and NAC intake were measured 4 wk after implementation of the diets (just prior to behavioral assessments) (**Figure 1**). Mice were 16–20% heavier at ages 12 and 24 mo than at age 6 mo (Figure 1A), supported by a main effect of Age ($P < 0.01$). At any age, NAC supplementation did not affect body weights ($P = 0.79$). At age 24 mo, the mice ate 20% more food than at ages 6 and 12 mo (Figure 1B). Moreover, the mice fed NAC consumed less food than did the control mice. These observations are supported by main effects of Age and Diet on food intake ($P < 0.01$ for all). NAC consumption was estimated to be 318 mg · kg^{-1} · d^{-1} for the 6- and 24-mo-old groups and 285 mg · kg^{-1} · d^{-1} for the 12-mo-old group, leading to a significant main effect of Age ($P = 0.011$; Figure 1C).

Behavioral assessments

Motor functions.

There was no difference between any of the groups for forward locomotion (**Figure 2**A) and rearing activity (Figure 2B), which was supported by a lack of significant main effect of Age or Diet, or interaction of Age and Diet on forward locomotion and rearing activity in the mice ($P > 0.29$ for all). Latency to initiate walking was affected by age in the control mice, specifically between ages 12 and 24 mo; however, there was no effect of age in the NAC-supplemented mice (**Figure 3**A). A 2-way ANOVA supported this observation with significant main effects of Age and Diet ($P < 0.03$ for all), but no significant interaction of Age and Diet ($P = 0.32$). The delay to turn in a dead-end alley increased with age, regardless of the diet, specifically between ages 12 and 24 mo (Figure 3B), supported by a significant main effect of Age ($P < 0.001$). NAC-supplemented young mice took shorter latency to turn 180° than the controls, whereas the NAC-supplemented old mice seemed to take longer than their age-matched controls, although the difference was not significant (Figure 3C). This observation led to a significant main effect of Age ($P = 0.028$), and an interaction that did not reach significance ($P = 0.050$). Latency to tread (musculoskeletal reflex) was longer in 12- and 24-mo-old mice than in 6-mo-old mice regardless of diet (Figure 3D), supported by a main effect of Age ($P < 0.001$). Latency to fall from the wire was shorter in the 24-mo-old mice than

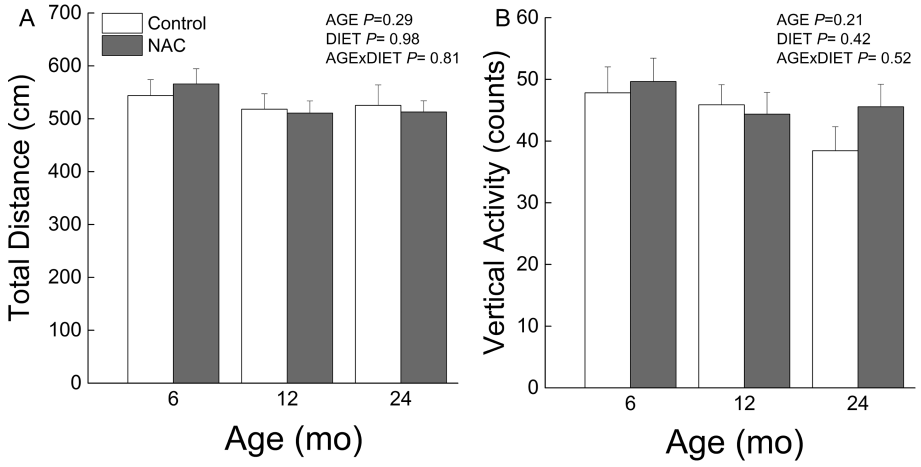

FIGURE 2 Effect of age and NAC supplementation on total distance (A) and vertical activity (B) in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM ($n = 29$–32). Labeled means without a common letter differ, $P < 0.05$. NAC, *N*-acetyl cysteine.

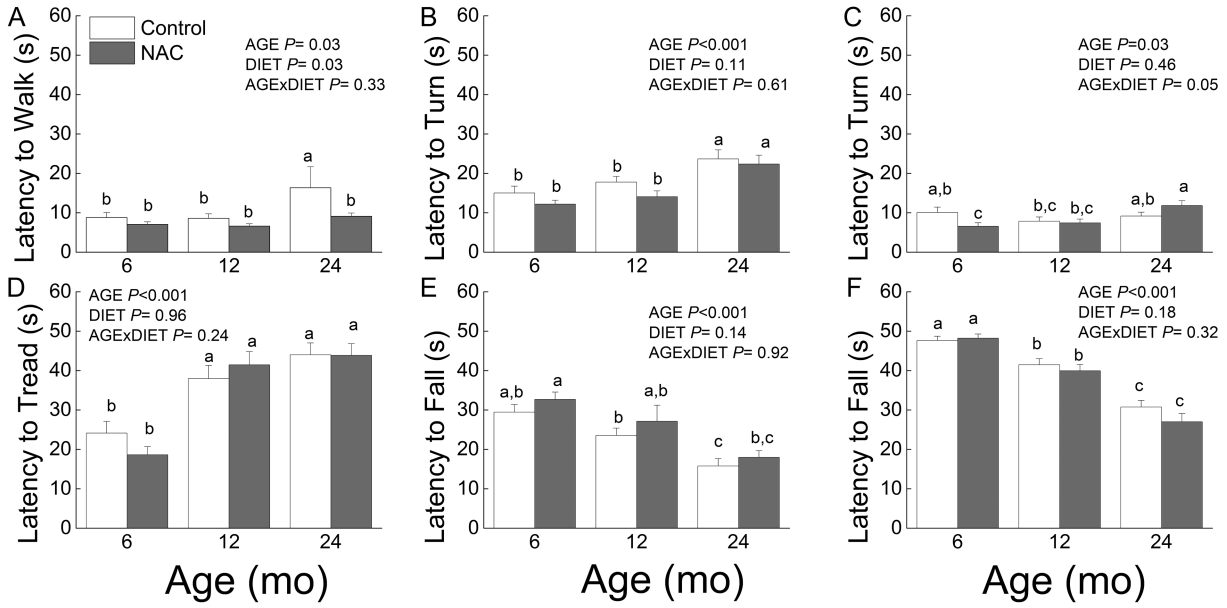

FIGURE 3 Effect of age and NAC supplementation on walk initiation (A), alley turn (B), negative geotaxis 180° (C), wire-suspension (D, E), and bridge-walking (F) in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM ($n = 29–32$). Labeled means without a common letter differ, $P < 0.05$. NAC, *N*-acetyl cysteine.

in the 6- and 12-mo-old mice regardless of diet (Figure 3E), supported by a main effect of Age ($P < 0.001$). Latency to fall from a suspended bridge was shorter in the 12- and 24-mo-old mice than in the 6-mo-old mice regardless of diet (Figure 3F), supported by a main effect of Age ($P < 0.001$).

Coordinated running.

Each group of mice increased their running performance over the first 7 training sessions, and middle-age and old mice took shorter latencies to fall than the 6-mo-old mice, supported by a main effect of Age over the 7 sessions and an interaction between Session and Age ($P < 0.05$ for all; data not shown). The plateau level of performance reached at the end of training depicted in **Figure 4** was also decreased with age. NAC intake increased the level of performance only at 6 mo of age. A 2-way ANOVA only revealed a main effect of Age ($P < 0.05$).

Spatial learning and memory.

All groups increased in efficiency (decreased swim speed independent path length) over the course of 9 sessions and learned to locate the platform (**Figure 5**A). Young and middle-age mice learned to locate the platform better than the old ones, regardless of diet. When we aggregated the data into a learning index (average of sessions 2–4, a smaller number means better performance; Figure 5B), there was a 29% increase in path length taken by the 24-mo-old mice compared with the 6-mo-old mice, regardless of diet. These observations were supported by a main effect of Age and an interaction of Session and Age ($P < 0.001$ for all).

Probe index, a measure of spatial bias, reflects the amount of time spent in the platform location when the platform is removed and was measured at the end of the acquisition phase (**Figure 6**A) and 1 wk later for retention (Figure 6B). The probe index was not affected by age in the control groups; however, there was an age effect in the NAC-supplemented groups due to the young NAC-supplemented mice spending more time in the platform location than any other groups. A 2-way ANOVA revealed a main effect of Age ($P = 0.033$); however, there was no main effect of Diet or an interaction of Diet and Age ($P > 0.13$ for all). While the 6- and 12-mo-old mice retained information on the platform location after a 1-wk delay (Figure 6B), the 24-mo-old mice spent less time in the area than during the last session of acquisition. There was an age-related decline in the probe index, reflected by a significant main effect of Age ($P < 0.001$).

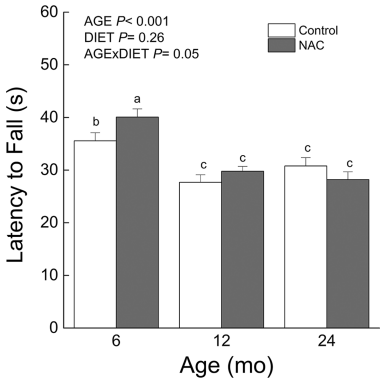

FIGURE 4 Effect of age and NAC supplementation on maximum coordinated running performance in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM ($n = 29–32$). Labeled means without a common letter differ, $P < 0.05$. NAC, *N*-acetyl cysteine.

Discriminated avoidance.

The average numbers of trials taken to reach a criterion of performance for each experimental group during acquisition and the 2 reversal sessions are depicted in **Figure 7**. There was an age-related increase in the number of trials taken to reach criterion during acquisition but no effect of diet (Figure 7A), supported by a main effect of Age ($P = 0.006$) and no main effect of Diet ($P = 0.68$). During the first reversal session, the control 12-mo-old mice took more trials to reach criterion than the 6-mo-old mice ($P = 0.05$), whereas for the NAC-supplemented mice it was the 24-mo-old mice. This observation was supported by an interaction between Age and Diet ($P = 0.044$). During the last reversal session, all mice

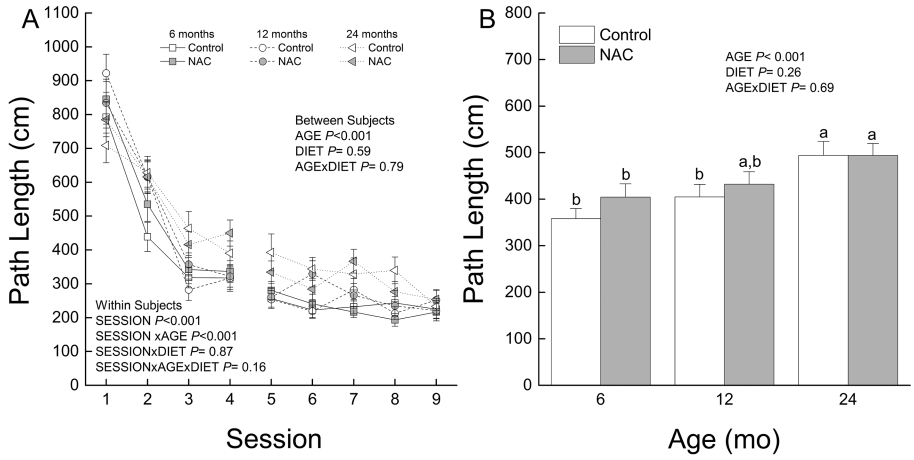

FIGURE 5 Effect of age and NAC supplementation on path length across sessions (A), and learning index (B) in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM ($n = 29–32$). Labeled means without a common letter differ, $P < 0.05$. NAC, *N*-acetyl cysteine.

performed better than during the first reversal session, and an age-related effect was apparent and not dependent on the diet. A 2-way ANOVA revealed only a significant main effect of Age ($P = 0.004$).

Discussion

The main findings of this study were that supplementation with NAC resulted in the following: *1*) improved motor coordination and spatial memory in 6-mo-old mice, but not in 12- or 24-mo-old mice; *2*) impaired cognitive flexibility in 24-mo-old mice; *3*) reversed age-related declines in walking initiation; and *4*) had no effect at any age on body weight, reflexes, balance, and learning.

Age-related motor and cognitive declines were evident based on results from most of the tests administered in this study and as reported previously (15, 33, 36, 37); however, NAC supplementation did not reverse any of the age-related impairments, and even exacerbated age-associated impairments in cognitive flexibility.

The outcomes of the present study contrast with previous reports on the effect of NAC on brain function, although differences in research methodology can be identified, such as duration of treatment, strain and sex of mice, and behavioral test utilized. Although our data suggest a lack of effect of NAC on age-related impairments in motor and cognitive function, Martinez et al. (28) reported that long-term NAC supplementation (6 mo compared with 1–2 mo in our study), at the same concentration as used in our study, partially restored memory measured with the use of a passive avoidance paradigm. The length of treatment in our study was chosen based on previous experiments indicating that antioxidants or caloric restriction interventions result in improved functional outcomes (38–41). Other differences between studies could explain the differential outcomes, notably the use of different strains and sex (OF-1 female compared with C57BL/6 male mice). It has been shown that strains respond differently to various anti-aging interventions and that there are sex differences in response to treatments (41–46). The present study, in contrast to others, has employed an exhaustive test battery to provide a wider view of the effect of NAC on overall brain function. Previous studies have focused on selected cognitive domains and provided only a limited view on the effect of NAC on brain function (27, 28). Furthermore, most previous studies of NAC intake focused on models of premature aging (20, 27). These studies suggest that under aggravated oxidative stress dysregulation and accelerated aging, NAC supplementation

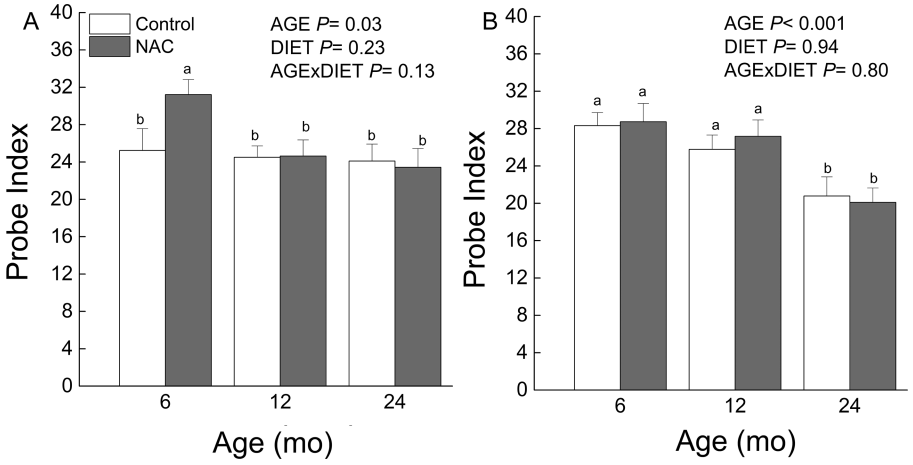

FIGURE 6 Effect of age and NAC supplementation on spatial bias measured as a probe index at completion of water maze training (A) and 1 wk later (B) in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM ($n = 29–32$). Labeled means without a common letter differ, $P < 0.05$. NAC, *N*-acetyl cysteine.

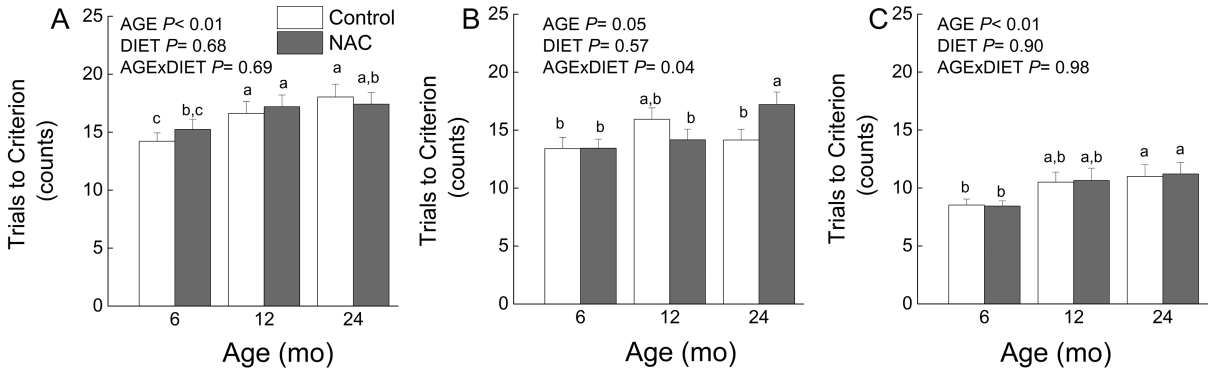

FIGURE 7 Effect of age and diet on number of trials taken to reach criterion in the discriminated active avoidance for acquisition (A), reversal 1 (B), and reversal 2 (C) in 6-, 12-, and 24-mo-old male mice. All values are the means ± SEM (n = 29–32). Labeled means without a common letter differ, $P < 0.05$.

may be a beneficial therapeutic. Most of the studies have also studied NAC in association with other antioxidants such as lipoic acid, thioproline, and vitamins E and C (24, 27, 47). All these differences between the current study and previous ones can account for the observed contrast in outcome in response to NAC supplementation. To date, the present study is the only one to have examined the effect of a 4- to 10-wk NAC supplementation alone on a multitude of motor and cognitive domains in a mouse model of aging.

Interestingly, performance on some aspects of motor (maximal performance on the coordinated running task) and cognitive (probe index in the Morris water maze task) function was improved in young mice following NAC supplementation. The presence of enhanced learning in young but not in old mice might be due to a differential response from the glutamatergic system. Synaptic transmission in models of learning and memory is dependent upon proper glutamate transmission and therefore redox balance (48), and it has been hypothesized that NAC supplementation can restore extracellular glutamate concentrations via the cysteine/glutamate transporter in models of addiction (49). One can speculate that NAC supplementation in the young mice led to an increase in cysteine concentration, thereby enhancing glutamatergic transmission, critical to the maintenance of a redox balance and improvement of brain function (50). However, in the aged mice, this effect is not perceived due to age-related alteration of the glutamatergic transmission, such as decreased glutamate synthesis and changes in glutamate metabolism along with accumulation of advanced glycation end-products (51). These outcomes suggest that the timing of intervention is of the utmost importance when studying interventions to delay the effects of aging. In this particular study, it is plausible that NAC was implemented too late, and was unable to reverse the age-associated impairments already present in the mice. The differential response suggests that the adaptive changes of an old brain are sufficient to block the effectiveness of interventions that are successful in young.

Based on the human:mouse body surface area ratio of 12:3, the human dose equivalent for the NAC dose administered to the mice in this study would be ~1680 mg/d (52). Nutritional supplement guidelines advise an intake of 1000 mg/d for a 70-kg individual, and therefore the dose given in this study is ~68% higher than the dose found in supplements, and similar to the dose used in some of the clinical trials studying the effect of NAC on Alzheimer disease, Parkinson disease, and neuropathic pain (53, 54). It would be of interest to determine whether a smaller dose, within the range of that found in supplements, could have a similar effect in young animals. However, it seems unlikely that a higher dose would lead to beneficial effects in aged animals, which was the initial objective of this study.

Previous studies have not demonstrated that oral administration of high doses of NAC produces adverse effects (3). Meta-analyses have determined that NAC supplementation is well tolerated by patients with chronic obstructive pulmonary disease, cystic fibrosis, and older individuals; however, there is some risk of patients exhibiting a higher propensity for bleeding (54). NAC supplementation was found by Flurkey et al. (55) to increase lifespan in male genetically heterogeneous mice, but was associated with a substantial decrease in body weight, and the authors attributed the effect on lifespan to a caloric restriction–like effect rather than an effect of NAC. In our study, we did not observe an effect of NAC supplementation on body weight or food intake; however, we used a daily dose that was half the lower dose used in the Flurkey et al. study.

Although the effects of NAC are postulated to occur via replenishment of GSH due to it being a precursor of glutathione, some studies have suggested that in the brain NAC supplementation does not affect redox status but rather attenuates oxidative damage in the synaptic mitochondria of the brain (28), suggesting a direct antioxidant effect. Based on the behavioral outcomes, we can speculate that NAC does reach the brain to elicit some effect, and others have reported NAC's ability to cross the blood-brain barrier (56), although it has a relatively low bioavailability due to extensive first-pass metabolism (57). The differential outcomes based on age may have been due in part to differences in bioavailability, suggesting that a more bioavailable derivative of NAC might offer better protective effects, especially in the old. One such compound, *N*-acetyl cysteine amide form, exists and has shown promise in models of traumatic brain injury; however, very little is known regarding its safety and mechanism of action.

In summary, our present findings support the conclusion that although short-term (4–10 wk) NAC supplementation at a high dose can enhance cognitive and motor function in young mice, it was ineffective in the amelioration of age-related impairments.

Acknowledgments

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